Exhibit 1

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Identification and Procaryotic Expression of the Gene Coding for the Highly Immunogenic 28-Kilodalton Structural Phosphoprotein (pp28) of Human Cytomegalovirus

H. MEYER,[1] A. T. BANKIER,[2] M. P. LANDINI,[3] C. M. BROWN,[2] B. G. BARRELL,[2] B. RÜGER,[1] AND M. MACH[1]*

Institut für Klinische und Molekulare Virologie der Universität Erlangen-Nürnberg, Loschgestrasse 7, 8520 Erlangen, Federal Republic of Germany[1]; Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom[2]; and Istituto di Microbiologia, Policlinico S. Orsola, Bologna, Italy[3]

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Human cytomegalovirus contains a structural polypeptide that is 28 kilodaltons in apparent molecular size and is reactive in Western blot (immunoblot) analysis with the majority of human sera. The gene coding for this polypeptide was mapped on the genome of human cytomegalovirus strain AD169. A monoclonal antibody specific for the 28-kilodalton polypeptide was used to screen a cDNA library constructed from poly(A)$^+$ RNA of human cytomegalovirus-infected cells in the procaryotic expression vector lambda gt11. Hybridization of cDNA with cosmid and plasmid clones mapped the gene to the *Hin*dIII R fragment. The gene was transcribed into a late 1.3-kilobase RNA. The nucleotide sequence of the coding region was determined. Parts of the 28-kilodalton polypeptide were expressed in *Escherichia coli* as hybrid proteins fused to β-galactosidase. In Western blots these proteins were recognized by human sera. Antibodies raised against the hybrid proteins reacted specifically with the viral antigen in immunoprecipitations and Western blots. In vitro phosphorylation of HCMV virions and immunoprecipitation showed that the 28-kilodalton polypeptide was phosphorylated.

Human cytomegalovirus (HCMV), a ubiquitous member of the herpesvirus family, can be associated with a wide spectrum of disease, particularly in immunocompromised persons. The factors that are responsible for this wide spectrum of clinical manifestation of HCMV infections are largely unknown. The route of infection and variation in the strain of infecting virus may influence the development of disease. Host immunological functions clearly play an important role in the control of infection. To more fully understand the importance of the immune response in limiting the severity of HCMV infections, it is necessary to first characterize the virus-encoded targets of this response. Serological diagnosis of active HCMV infection is mainly based on a significant rise in antibody levels detected by a complement-fixation test or an enzyme-linked immunosorbent assay. While highly useful in detecting an immune response to the virus, these assays do not determine the fine specificity of antiviral antibodies since each assay indiscriminately detects antibodies against multiple viral antigens. To give a more precise definition of the humoral immune response during active HCMV infection it is necessary to evaluate titers against defined antigens. HCMV is a highly complex virus encoding approximately 30 to 40 structural proteins and an unknown number of nonstructural polypeptides (for a review, see M. P. Landini and S. Michelson, Prog. Med. Virol., in press). Several laboratories have employed Western blotting (immunoblotting) analysis or immunoprecipitations to identify individual viral polypeptides that are recognized by human immune sera (14, 15, 19; B. Nowak, Ph.D. thesis, University Erlangen-Nürnberg, 1984). In these studies it was found that immune sera contain various amounts of antibodies against the majority of the structural components of HCMV. The immune response involved 15 to 25 proteins ranging in molecular size from 28 to 200 kilodaltons (kDa) (14, 37). However the intensity and frequency of the immune reaction was particularly high for four polypeptides of 150, 65, 58, and 28 kDa. Three of the proteins which elicited this antibody response were characterized. They corresponded to the basic phosphoprotein (pp150), the major matrix protein (pp65), and the major glycoprotein (gp58). As an initial step in studying the immunogenic potential of various structural polypeptides, we started to isolate the genes coding for these highly immunoreactive proteins. This information will be used to synthesize viral antigens via procaryotic or eucaryotic expression systems and to measure antibody levels in a defined system. The mapping and the characterization of the genes coding for pp150, pp65, and gp58 have been reported (9, 17, 24). The approach to the characterization of pp150 and gp58 was the isolation of clones coding for these polypeptides by procaryotic expression cloning. A cDNA library from poly(A)$^+$ RNA, isolated from HFF cells at late times in infection, was constructed in the vector lambda gt11 (17). In this system the cDNA is inserted into the 3' end of the *lacZ* gene. Expression of the gene results in the synthesis of a fusion protein of β-galactosidase and the respective foreign polypeptide which can be detected by monospecific antisera or monoclonal antibodies. Here we describe the identification and characterization of the gene coding for the fourth highly immunogenic structural polypeptide of HCMV, the 28-kDa polypeptide, which is also shown to be a phosphoprotein. We also show that a recombinant *Escherichia coli* protein, containing parts of pp28, can be used to detect HCMV antibodies in human sera.

MATERIALS AND METHODS

Virus, cell culture, and virion purification procedures. HCMV strain AD169 was propagated in human foreskin fibroblasts by standard methods. Extracellular virus was purified from the tissue culture supernatant through a glycerol-tartrate gradient (31).

Recombinant plasmids. Plasmids pRR1, pCMS007, and

* Corresponding author.

pCM5009 were described previously (25). Plasmid p271 was generated by inserting the 500-base-pair (bp) *Eco*RI-*Sma*I fragment of plasmid pHM7 into pEX-2 (29). pHM7 contains the 500-bp *Kpn*I-*Sma*I fragment inserted into M13mp11 (nucleotides 1443 through 1936).

Construction and screening of the cDNA library. The construction and screening of the lambda gt11 library have been described in detail (9, 17, 36).

Sequence analysis of the viral DNA. Sequencing of lambda gt11 cDNA and viral DNA was performed by the chain termination method (26). The cDNA was subcloned into M13mp11 before sequence analysis. Sequence analysis of *Hin*dIII R fragment was carried out using the M13-chain termination method (26) with the random strategy of Bankier and Barrell (2). The fragment was excised from the plasmid, which was kindly provided by J. Oram and P. Greenaway. The sequence was determined on both strands by the shotgun method, and each base was determined an average of 6.36 times. The DNA sequence was compiled with the DBOTIL program (27) and analyzed with the ANALYSEQ program (28).

Induction of fusion proteins. Fusion proteins of gt11 clones were produced as described previously (36). P271 fusion proteins were produced in *E. coli* pop2136 (29). The cells were grown to a density (A_{600}) of 0.2 to 0.3 at 30°C and induced by a quick temperature shift to 42°C. Synthesis of the fusion protein was allowed to continue for 90 min. After that the cells were harvested, lysed in sodium dodecyl sulfate (SDS)-gel sample buffer, and analyzed on SDS-polyacrylamide gel electrophoresis.

Protein gel electrophoresis and Western blot analysis. Polypeptides were denatured in SDS, electrophoretically separated on polyacrylamide gels, and transferred to nitrocellulose as described previously (13, 34).

Production of monospecific antisera against fusion proteins. *E. coli* extracts containing the fusion proteins were separated on preparative 8% polyacrylamide gels, stained with Coomassie brilliant blue, and partially destained. The band corresponding to the fusion protein was cut out and frozen in liquid nitrogen. The frozen polyacrylamide gel slices were pulverized in a Dysmembrator (Fa. Braun, Melsungen, Federal Republic of Germany), and the proteins were extracted by repeated incubations with 0.1 M $(NH_4)HCO_3$ (pH 9.5) containing 0.1% SDS. The combined eluates were lyophilized and used to immunize rabbits. Initial injections were administered with complete Freund adjuvant, and booster injections were administered with incomplete adjuvant at 4-week intervals. Antibody titers in sera were monitored in Western blots.

In vitro phosphorylation. Extracellular virions were purified from two 150-cm² culture flasks, washed three times with buffer A (0.1% Nonidet P-40, 20 mM $MgCl_2$, 50 mM Tris hydrochloride [pH 8.0]), and collected by centrifugation (30 min, 4°C, 20,000 × *g*). The protein content was determined in the virus suspension by the Bio-Rad assay. For the kinase reaction 10 μg of protein was incubated with 7 μCi of [γ-^{32}P]ATP and 200 μl of kinase buffer (buffer A containing 0.1% Nonidet P-40) for 1 h at room temperature. Then 10% of the reaction was precipitated with trichloroacetic acid, and the incorporation of radioactivity into the protein was determined. Usually about 500,000 cpm/μg of protein was incorporated.

Immunoprecipitation. Immunoprecipitations were performed as described previously (19), with minor modifications. The lysates were precleared by incubation with normal mouse or rabbit serum, and Formalin-fixed *Staphylococcus aureus* cells (Behringwerke, Marburg, Federal Republic of Germany) at 4°C for 30 min. The lysates were then clarified by centrifugation at 10,000 × *g* for 5 min.

RNA extraction and Northern blots. For the preparation of RNA, 70 to 80% confluent monolayers of HFF cells were infected with HCMV strain AD169. Whole cell RNA was extracted from infected cells as described previously (3). For immediate-early RNA, the cells were maintained in medium containing cycloheximide (50 μg/ml) for 60 min before infection with 5 to 10 PFU per cell. The cells were infected for 14 h in medium containing cycloheximide, and RNA was prepared. For early RNA, cells were maintained in medium containing 100 μg of phosphonoacetic acid per ml from 3 to 22 h after infection. For late RNA, cells were harvested and RNA was prepared at 72, 96, and 120 h after infection. The RNA was fractionated on 1.5% agarose–2.2 M formaldehyde gels. Human 18S and 28S rRNA and bacterial 16S and 23S RNAs were used as size markers. RNA was transferred to nitrocellulose membranes as described previously (33).

RESULTS

Isolation of a cDNA clone coding for pp28. In our previous studies monospecific antibodies against polypeptides partially purified on preparative polyacrylamide gels were used. The purification of the 28 kDa polypeptide, however, was not feasible by this method since it is not a constituent which is easily identified in preparative gels. We therefore used the monoclonal antibody P2G11. In Western blots this antibody reacts with a protein of 28 kDa in infected cells and purified virions (22). The antigen recognized by the monoclonal antibody P2G11 appeared to correspond to the immunogenic 28-kDa polypeptide recognized by immunoglobulin G present in human sera (22).

A total of 150,000 recombinant gt11 phages were screened with P2G11. Two positive signals were obtained. One clone (designated BUML-1) was purified and characterized in detail. *E. coli* Y1089 cells were infected with the recombinant phages, and the synthesis of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside. In protein extracts of *E. coli* cells infected with BUML-1 an abundant protein of about 130 kDa was synthesized which was not present in *E. coli* cells infected with lambda gt11. Here a protein of 118 kDa, corresponding to β-galactosidase, was detectable. Both polypeptides were readily detectable in polyacrylamide gels after staining with Coomassie brilliant blue, suggesting a high synthesis rate. In Western blots the 130-kDa polypeptide of BUML-1 reacted exclusively with the monoclonal antibody P2G11 (data not shown). Proteins from lambda gt11-infected cells or fusion proteins from unrelated cDNA clones were not recognized. We took this as evidence that BUML-1 was synthesizing a hybrid protein of β-galactosidase and parts of pp28 of HCMV. To further substantiate this, DNA was prepared from plate lysates and the cDNA insert was cut out with *Eco*RI, the enzyme used in the original cloning procedure. In agarose gels stained with ethidium bromide a DNA fragment of about 270 bp was detected (data not shown). This fragment was radiolabeled with ^{32}P and hybridized in a dot-spot assay to DNA from eight cosmid clones spanning the whole HCMV genome (5). The cosmid pCM1058, containing the fragments *Hin*dIII-T, -R, and -E, hybridized to the cDNA. In a more detailed Southern blot analysis the complementary sequences were located within a 500-bp *Kpn*I-*Sma*I fragment at the left end of *Hin*dIII-R (Fig. 1A). Since *Hin*dIII-R is cleaved into tw *Kpn*I-*Sma*I fragments of equal size (Fig.

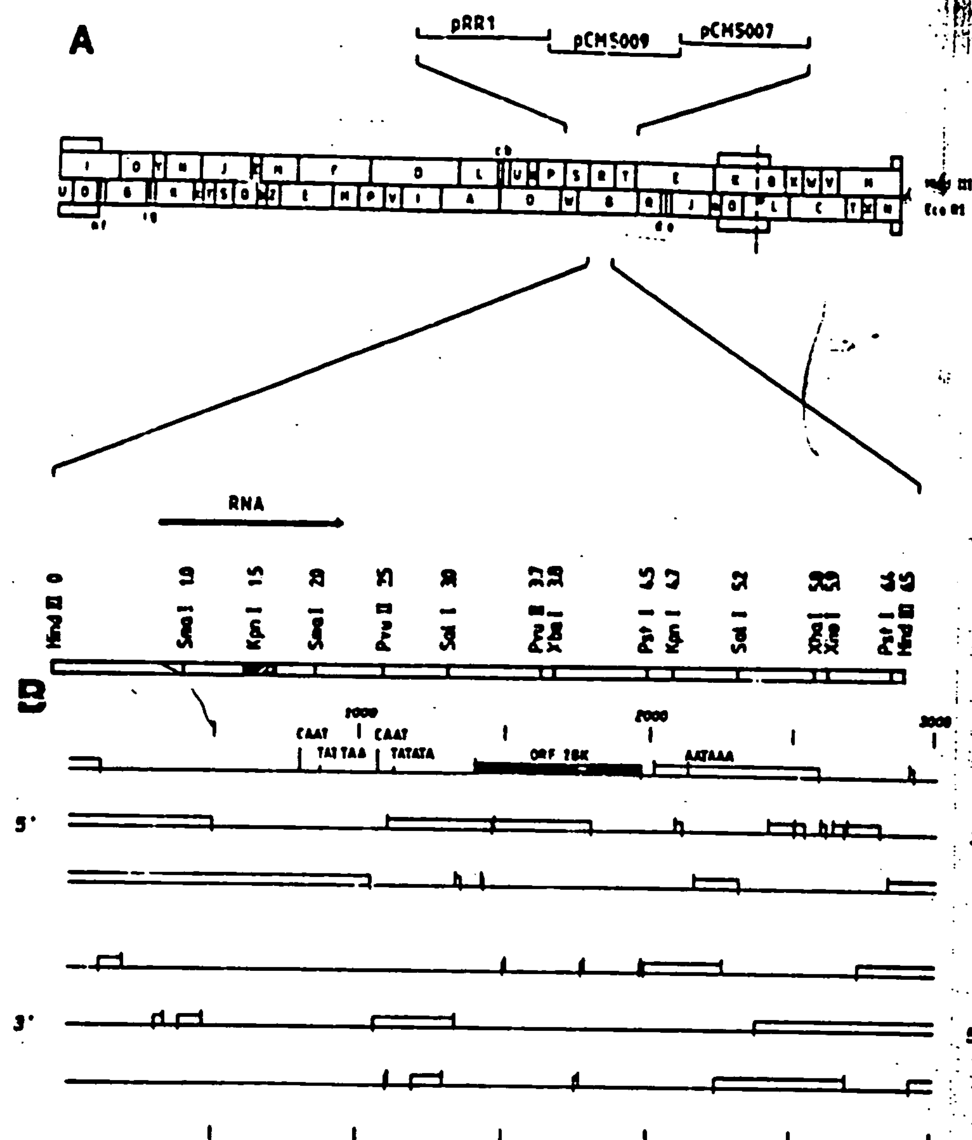

FIG. 1. Structure of the HCMV AD169 genome and localization of the pp28 DNA. (A) Schematic representation of the HCMV genome with the restriction maps for *Hin*dIII and *Eco*RI. The plasmids containing *Hin*dIII fragments S (pRR1), R (pCMS009), and T (pCMS007) are indicated on top of the map. The 6.5-kb *Hin*dIII R fragment is shown on an expanded scale below the HCMV genome. Only some relevant restriction endonuclease recognition sites are given. The localization of the cDNA of BUML-1 is marked by the hatched area. The orientation of transcription is indicated by an arrow. (B) Reading frame analysis of the first 3,000 nucleotides of *Hin*dIII-R (4). Bars above the horizontal line indicate start codons; bars below the lines indicate stop codons. The potential CAT and TATA sites and the poly(A) signal sequence are indicated.

1A), it was not possible to determine the exact location of the cDNA complementary sequences in this analysis. However, the two fragments can be distinguished by an *Sst*II recognition site located at nucleotide 1877. Taking advantage of this restriction site, it was possible to locate the cDNA to the right *Kpn*I-*Sma*I fragment in the genome orientation shown in Fig. 1A. To establish the nucleotide sequence the 270-bp cDNA fragment was subcloned into M13mp11 and sequenced by the chain termination method (26). The exact size of the cDNA was found to be 273 nucleotides (Fig. 2). It contained an open reading frame running through the entire fragment. The translation of 273 bp in addition to the *lacZ* gene would result in a polypeptide of approximately 130 kDa. The size of the fusion protein synthesized in BUML-1 is in good agreement with this theoretical value.

Genomic sequence of the region coding for pp28. The nucleotide sequence of a 3-kilobase (kb) segment within *Hin*dIII-R was determined by the dideoxy-chain termination

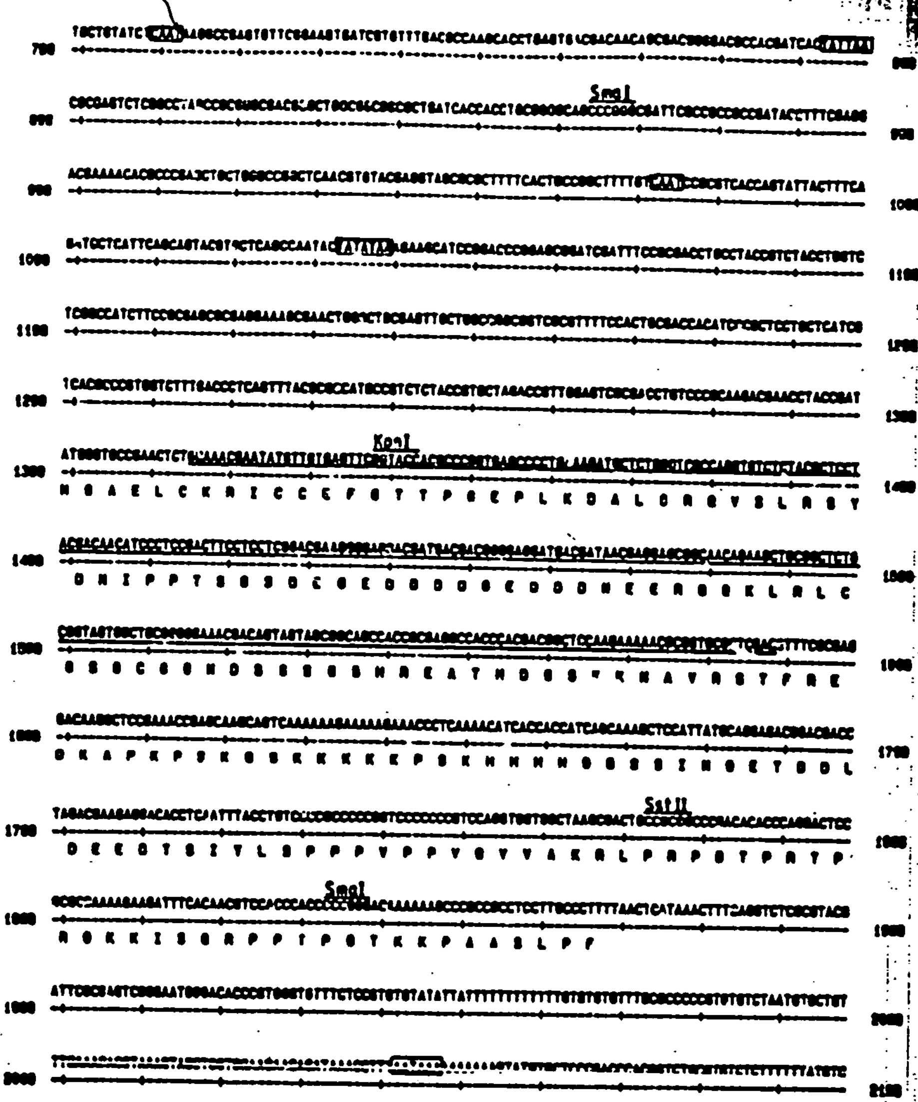

FIG. 2. Nucleotide sequence of the *Hin*dIII-R DNA segment encoding pp28. The *Hin*dIII site between fragments S and R is taken as zero. For the sake of uniformity with Fig. 1B the sequence has not been renumbered. The cDNA clone BUML-1 (underlined) corresponds to the virion DNA sequence between nucleotides 1415 and 1688. The TATA consensus sequences and the poly(A) signal sequence AATAAA are boxed. The amino acid sequence of ORF pp28 (Fig. 1B) is given in the one-letter code.

method. Figure 2 shows the sequence of nucleotides 800 to 2200. The *Hin*dIII site between fragments S and R is taken as nucleotide 0. The region of full homology between cDNA and genomic DNA extends from nucleotides 1415 to 1682. This determines the open reading frame (ORF) between nucleotides 1399 and 1967 as the coding region for pp28 if the transcript is not spliced (see below). The AUG codon at position 1399 follows the known rules for effective translational start sites (12). Analysis of the nucleotide sequence upstream region revealed two potential TATA box sequences at nucleotides 893 through 898 and 1133 through 1140. Both are preceded by potential CAAT boxes 83 and 59 nucleotides upstream. However, there is another potential initiation codon that fulfills the rules of K zak between the potential prom ter sequences and the initiation codon in the pp28 reading frame. This ATG at position 1336 has a purine at position −3 but is followed 7 codons later by a termination codon. A potential polyadenylation signal (AATAAA) is located at nucleotides 2144 through 2149. The ORF pp28 is surrounded by two ORFs running in different directions (Fig. 1B). The left ORF starts within *Hin*dIII S and extends to nucleotide 1046 (Fig. 2). The right ORF is located at the opposite strand ending at nucleotide 2238. It is worth noting that both left and right ORFs show homology to reading frames BGLF5 and BBRF3 of Epstein-Barr virus. On the Epstein-Barr virus genome the two reading frames are arranged in a nearly identical fashion (1). N homology to other published herpesvirus sequences was found for pp28. The numerical molecular size of the polypeptide encoded by ORF pp28 is 20,933 Da. This is a clear discrepancy with the

FIG. 3. Identification of transcripts homologous to BUML-1 in infected cell RNA. Whole cell RNA was isolated under immediate-early (lane 2), early (lane 3), and late (lane 4) conditions and fractionated on a 1.5% agarose gel containing formaldehyde. The gel was blotted onto nitrocellulose and probed with the radiolabeled 275-bp cDNA fragment of BUML-1. Lane 1 contains RNA from mock-infected cells. Each lane contains 15 μg of RNA. Fragment size is given in kilobases.

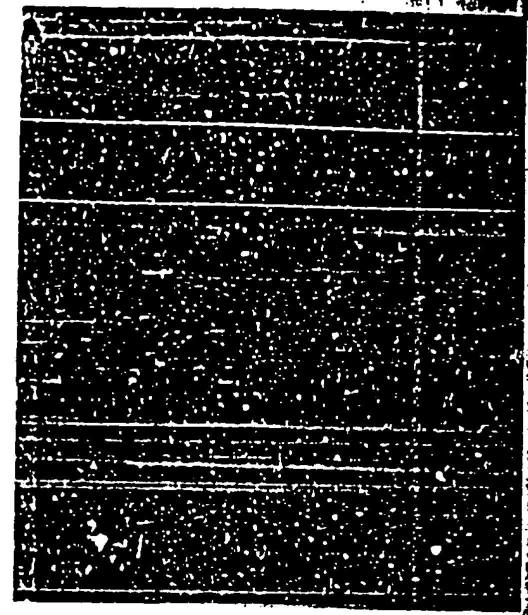

FIG. 4. Recognition of the β-galactosidase-pp28 hybrid protein by human sera. (A and B) *E. coli* extracts were separated by SDS-polyacrylamide gel electrophoresis on a 8% acrylamide gel. After transfer to nitrocellulose the membrane was cut, and one half was stained with amido black to control for the transfer (A). The second half was subjected to the immune reaction (B). Lanes (p271) protein extract from clone p271; (pEX2) protein extract from pEX2; (Std) molecular weight marker (Sigma Chemical Co.). Serum (code no. 54527) dilution was 1:750. (C) Proteins from purified virions or dense bodies were separated on a 10% acrylamide gel and subjected to a Western blot analysis with the same human serum samples as in A and B. Lanes: (V) virions; (Db) dense bodies. Serum (code no. 54527) dilution was 1:250. All sizes are given in kilodaltons.

size estimated from polyacrylamide gel electrophoresis. The difference between this and the theoretical value of about 7 kDa is most probably due to the phosphorylation (see below) and the unusual characteristics of the protein. Computer analysis by the method of Hopp and Woods (7) revealed that pp28 is an extremely hydrophilic protein. pp28 lacks tryptophan, the most hydrophobic amino acid, and contains only 1% phenylalanine and 1% tyrosine, whereas the hydrophilic residues arginine, aspartic acid, glutamic acid, and lysine make up 33% of the molecule. According to the hydrophilicity pattern the most hydrophilic regions are centered around amino acids 50 through 60 and 110 through 120. The monoclonal antibody P2G11 recognizes an epitope encoded by the cDNA between amino acids 7 and 95. This is consistent with the prediction of Hopp and Woods that hydrophilic regions are highly antigenic. An overestimation of the molecular size seems to be a phenomenon common to the phosphoproteins of HCMV. Both the basic phosphoprotein and the major matrix protein have apparent molecular sizes of 150 and 65 kDa when estimated by analysis on SDS-polyacrylamide gel electrophoresis. The theoretical values, however, calculated from computer analysis are 113 and 61 kDa, respectively (9, 24).

Analysis of the transcript and time of appearance. In Northern blot analysis the size of the pp28-specific transcript was determined. Total RNA from AD169-infected cells was isolated under immediate-early, early, and late conditions. The RNAs were separated on a 1.5% agarose denaturing gel and probed with the ^{32}P-radiolabeled cDNA BUML-1 fragment. A RNA species of 1.3 kb was the most prominent signal (Fig. 3). This transcript was present only in RNA preparations isolated late in infection. The 1.3-kb RNA was encoded entirely within the *Hin*dIII R fragment. In Northern blots with the surrounding fragments *Hin*dIII-T and -S no RNA species of this size could be detected at late times (data not shown). The 1.3-kb RNA was abundant late in the infectious cycle. An additional late RNA of 1.5 kb was also detected within the *Hin*dIII R fragment. This RNA is transcribed from coding sequences downstream of ORF pp28, most probably from the right ORF (H. Meyer, M. Mach unpublished). Northern blot analysis with single-stranded cDNA probes confirmed that the direction of transcription for the 1.3-kb RNA was from left to right in the genome arrangement shown in Fig. 1A (data not shown).

Expression cloning of pp28. To express a large part of pp28 for immunological studies, plasmid p271 was constructed (see Materials and Methods). The vector contains a 500-bp *Kpn*I-*Sma*I fragment of pp28 fused to the cro-β-galactosidase gene of pEX-2 (29). The *Kpn*I-*Sma*I fragment codes for 87% of pp28. Fourteen amino acids of the amino terminus and 11 of the carboxy terminus are lacking in clone p271. The synthesis of the hybridprotein is controlled by the P_R promoter of phage lambda. Upon induction, a protein of 136 kDa accumulated to high levels in *E. coli* cells carrying the p271 plasmid (Fig. 4A). This polypeptide was recognized specifically by monoclonal antibody P2G11, indicating that the p271 construct carried the HCMV sequences in the right orientation and reading frame.

pp28-β-galactosidase fusion protein recognized by human sera. To test whether HCMV polypeptides synthesized in *E. coli* could be used as antigens for diagnostic purposes, Western blots with protein extracts of clone p271 were carried out. In the first set of experiments 14 HCMV-positive human serum samples and 6 negative serum samples were analyzed. Since human sera may contain antibodies against *E. coli* proteins and especially β-galactosidase, all serum samples were preincubated with an extract of *E. coli* cells expressing the cro-β-galactosidase protein. The same extracts were also run as controls. An example of a Western blot analysis is shown in Fig. 4. The serum weakly reacted with the pp28 polypeptide when gradient-purified virus was used as the antigen. The reactivity was below detection

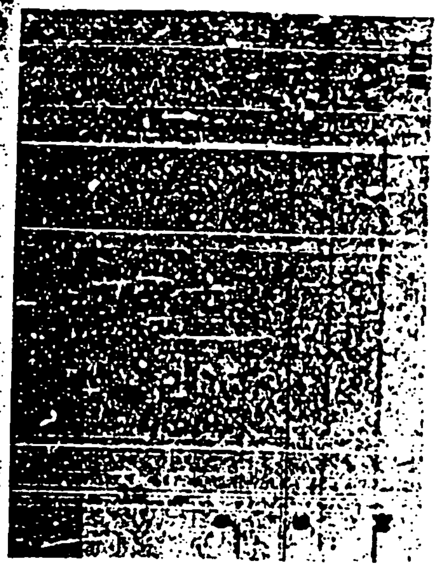

FIG. 5. Detection of pp28 with antisera against recombinant proteins. Proteins from extracellular virions were separated by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel. After transfer to nitrocellulose the membranes were cut and subjected to immunereactions with various sera (A). (B) Amount of protein transferred to nitrocellulose, stained with amido black. Lanes: (Vir) extracellular HCMV virions; (M) molecular weight markers; (P2G11) monoclonal antibody P2G11 dilution 1:250; (p aBUML, p a271) preimmune rabbit sera; (aBUML, a271) rabbit sera made against the fusionprotein of BUML and p271, respectively. All sizes are given in kilodaltons.

limits when purified dense bodies were used (Fig. 4C). The p271 fusion protein, however, was strongly recognized by the serum even at a fourfold-higher dilution (Fig. 4B). This might be due to the higher amount of antigen present in the p271 protein extract. The preincubation of the human sera with the *E. coli* extract efficiently eliminated the problems which may arise from anti-*E. coli* antibodies. Of the 14 human serum samples, 10 recognized pp28 in purified virions. The same serum samples also reacted with the p271 fusion protein, indicating that a recombinant protein can substitute for the authentic antigen. No reaction was seen with all of the HCMV-negative sera.

An antiserum was also raised against the recombinant protein. Protein extracts from p271 cells were separated on a preparative 10% polyacrylamide gel, and the 136-kDa band was cut out after staining with Coomassie brilliant blue. The protein was eluted from the gel and used to immunize rabbits. The reactivity of the serum was monitored in Western blots by using purified virions as antigens. After one booster injection the rabbit sera reacted with a polypeptide which showed the same apparent molecular weight as pp28 (Fig. 5). The fast immune response again proves the high immunogenicity of the pp28 protein. Moreover, a serum raised against the BUML fusion protein also recognized the pp28 and did not show any reactivity to other HCMV proteins (Fig. 5). The specificity of the recombinant sera represents additional proof that the identified reading frame within the *Hin*dIII R fragment in fact encodes the 28-kDa polypeptide of HCMV. Neither recombinant serum was able to neutralize HCMV strain AD169 grown in tissue culture (data not shown).

FIG. 6. SDS-polyacrylamide gel electrophoretic analysis of immune precipitated proteins from in vitro-phosphorylated HCMV virions. Monoclonal antibody P2G11 (lane 3), a271 rabbit serum (lanes 4 and 5), a rabbit serum against non infectious enveloped particles (lane 1), and a preimmune rabbit serum (lane 2) were used as sources of precipitated antibodies. Precipitated proteins were eluted in the presence of β-mercaptoethanol, analyzed on a 10% acrylamide gel, and exposed to X-ray film. Lane 6 contains HCMV proteins labeled in vitro with [γ-^{32}P]ATP. Lane 7 contains a ^{14}C-labeled protein mixture. Molecular sizes (in kilodaltons) are shown on the right.

Immunoprecipitation of pp28 with different antibodies. Previous studies have described a phosphoprotein (19; Nowak, Ph.D. thesis), a glycoprotein (20), and a capsid protein (8) with molecular weights in the range of 28 kd. To test whether pp28 described in this work was identical with one of these polypeptides, we investigated a potential posttranslational modification of the protein. Digestion of virion proteins with endoglycosidases did not alter the mobility of pp28, suggesting that the protein was not glycosylated to a great extent (data not shown). To analyze a potential phosphorylation of pp28, extracellular HCMV particles were phosphorylated in vitro. HCMV particles have been shown to contain an associated protein kinase which is capable of phosphorylating in vitro the same polypeptides which are phosphorylated in vivo (23). The presence of a virion-associated protein kinase is typical of enveloped viruses and has been reported for many members of the herpesvirus group (30, 32). Extracellular HCMV particles were collected from tissue culture supernatants 72 h postinfection. The HCMV particles were separated into virions, noninfectious enveloped particles (NIEPS), and dense bodies by a glycerol-tartrate gradient. The fraction containing virions was collected and used for the in vitro phosphorylation. Five proteins of 150, 71, 65, 36, and 28 kDa were most intensely labeled (Fig. 6). They correspond to the basic phosphoprotein (pp150), upper and lower matrix proteins (pp71 and pp65), assembly protein (pp36), and the 24-kDa phosphoprotein, respectively, as described by Roby and Gibson (23). The presence of the 36-kDa assembly protein indicates that the virus preparation was not completely free of NIEPS, since this polypeptide is an exclusive constituent of NIEPS (8). In agreement with previous studies (23), we also observed that the 71-kDa upper matrix protein was phosphorylated better in vitro when compared with the lower matrix

protein of 65 kDa. The in vitro-phosphorylated proteins were immunoprecipitated with monoclonal antibody P2G11 and the rabbit antiserum raised against the p271 fusion protein. Both antibodies precipitated a protein of 28 kDa (Fig. 6). The same polypeptide, was also precipitated, although to a much lesser extent, by a rabbit serum raised against NIEPS, (Fig. 6). In addition the NIEPS antiserum precipitated phosphorylated proteins of 150, 80, 71, 65, 52, and 36 kDa. The reactions are highly specific, since a rabbit preimmune serum did not precipitate any of the phosphorylated proteins. These data strongly suggest that the 28-kDa structural protein of HCMV, which is encoded by the *Hin*dIII R fragment, is a phosphoprotein.

DISCUSSION

In this report we describe the identification of the gene coding for the immunogenic 28-kDa phosphoprotein by using a monoclonal antibody.

After the initial isolation of immunoreactive lambda gt11 HCMV plaques, the genomic region was localized by hybridization analysis with available cosmid and plasmid clones of HCMV DNA. The coding sequence for pp28 is located in the long unique segment of the viral genome within the left end of the *Hin*dIII R fragment. In this area three ORFs are located. The nucleotide sequence comparison between cDNA and genomic DNA established that the small ORF codes for pp28. Our data exclude the possibility that pp28 is derived from a spliced transcript containing parts of the left ORF and the small adjacent ORF. The pp28 cDNA spans the gap which would have to be spliced out to create an ORF. The pp28 gene is transcribed into a 1.3-kb mRNA which is present exclusively late in the infectious cycle. In this study we did not attempt to map this RNA precisely. A detailed analysis of the transcripts originating from this part of the *Hin*dIII R fragment will be presented elsewhere (R. Lehner and M. Mach, manuscript in preparation). With the accumulating sequence information on all human herpesviruses it becomes apparent that some regions of the viral genomes show extensive homology. These similarities are reflected in the sequence of the proteins encoded as well as the relative organization of the genes. Examples are the coding regions for the DNA polymerase and the major glycoprotein (1, 6, 11). There are, on the other hand, a number of HCMV genes which do not show homology to those of other herpesviruses. Examples are the 150-kDa basic phosphoprotein, the 65-kDa major matrix protein, and the entire short unique region (35). In pp28 a different pattern seems to exist. An unique gene is located interspersed between reading frames which are conserved between distantly related herpesviruses.

Recently, Martinez and St. Jeor (18) described the isolation of a lambda gt11 clone which also maps within the *Hin*dIII R fragment. The fusion protein synthesized by this clone is recognized by human sera. Antibodies raised against the fusion protein recognize a 19-kDa structural polypeptide which is transcribed from a small RNA of about 1.5 kb. Since no precise mapping or sequencing data are given, it is not clear whether both proteins are identical, especially since the *Hin*dIII R fragment codes for a number of immunogenic structural proteins (R. Lehner and M. Mach, unpublished data).

In several studies HCMV polypeptides in the range of 28 kDa have been described. In four reports either a posttranslational modification or the localization of the protein within the virus has been investigated. Roby and Gibson (23) as well as Nowak et al. (19; Nowak, Ph.D. thesis) described phosphoproteins of 24 and 29 kDa, respectively. They suggested that the proteins are located in the matrix rather than the capsid or the envelope. Pereira et al. (21) described a monoclonal antibody which in immunoprecipitations reacts with a 25-kDa glycosylated protein, a member of the gD family, in infected cell extracts. Irmiere and Gibson (8) have identified a 28-kDa protein that is present in both the A and B capsids of different HCMV strains. Our results suggest that the 28-kDa protein corresponds to the phosphorylated matrix protein. Consistent with this suggestion are immunoelectron microscopy studies which localized the protein recognized by the monoclonal antibody P2G11 on the outline of cytoplasmic viral capsids (16).

The fact that pp28 is recognized by the majority of human sera turns it into a candidate for a diagnostic reagent. The most reliable protein in this regard is the basic phosphoprotein (pp150). It is recognized by virtually all sera that we (10) and others (14) have tested and therefore represents a useful tool to assess the occurrence of a previous HCMV infection. Preexisting high anti-pp150 titers, on the other hand, may complicate the detection of a recurrent infection if the diagnostic procedure is based exclusively on this protein. Therefore, it seems desirable to include additional antigens in the evaluation of a serological HCMV test. It has been shown that together with pp65 and gp58, antibody titers against pp28 increase substantially during a recurrent HCMV infection (14). Therefore these four proteins (pp150, pp65, gp58, and pp28) might represent an antigenic complex sensitive enough to detect low levels of antibodies due to a past infection and able to detect changes in antibody titers due to a present infection. We have shown here that a recombinant protein synthesized in *E. coli* can substitute for the authentic pp28 antigen. Although the number of sera that we have tested is small, we believe that it is safe to assume that in general this polypeptide produced in *E. coli* can serve as a reliable antigen for the detection of antibodies against pp28. A study testing this hypothesis on a sufficient number of antisera is currently in progress. To avoid problems which could arise from antibodies against *E. coli* proteins, it would be advantageous to express pp28 unfused and to purify the protein. These preparations could then be used in enzyme-linked immunosorbent assays. So far we have not been able to produce sufficient quantities of pp28 in an unfused state.

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